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(54) **VESICULAR ADAPTOR AND USES THEREOF IN NUCLEIC ACID LIBRARY CONSTRUCTION AND SEQUENCING**

VESIKULÄRER ADAPTER UND VERWENDUNGEN DAVON IN DER NUKLEINSÄUREBIBLIOTHEKKONSTRUKTION UND -SEQUENZIERUNG

ADAPTATEUR VÉSICULAIRE ET SES UTILISATIONS DANS LA CONSTRUCTION ET LE SÉQUENÇAGE D'UNE BIBLIOTHÈQUE D'ACIDES NUCLÉIQUES

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Description

FIELD

[0001] The present disclosure relates to the field of biotechnology, in particular to use of an oligonucleotide vesicular adaptor for constructing a cyclic single-stranded nucleic acid library.

BACKGROUND

[0002] The second-generation sequencing technology, also known as Next-generation sequencing technology, is named with respect to the first-generation sequencing technology which is represented by the Sanger sequencing method. The second-generation sequencing technology is represented by Roche/454 Pyrosequencing, Illumina/Solexa polymerase synthesis sequencing and ABI/SOLiD ligase sequencing, and their common characteristic is high sequencing throughput. Compared with these mainstream sequencing platforms, the Complete Genomics (CG) sequencing platform with the highest throughput may produce 9.9TB of data in each run, and its output may reach 50 Gb per hour, which is 10-25 times that of the mainstream sequencing platforms. With respect to read length for haploidy, among the mainstream sequencing platforms, only the Illumina sequencer may achieve a read length of 8-10 kb for haploidy, while the CG sequencer may reach a read length greater than 99kb. In addition, the CG sequencer may achieve accuracy up to 99.999%, better than other commercial sequencers. Thus, compared with the mainstream sequencing platforms, the CG sequencing platform has unique advantages.

[0003] In the process of constructing a nucleic acid sequencing library, it is generally necessary to introduce an adaptor with a known sequence for sequencing. However, it has been reported that when an adaptor is ligated for library construction in known way that not only ligating efficiency is not high enough, but also many by-products at low level arise. In addition, as the CG sequencing platform adopts a cyclic single-stranded library for sequencing, linear double-stranded libraries constructed for the mainstream sequencing platforms are thus not suitable for the CG sequencers. However, as to the method for constructing the cyclic single-stranded library for nucleic acid sequencing, there has been no literature reported so far.

[0004] Based on above situation, an adaptor with high ligating efficiency and accuracy is urgently required to be developed in the related art.

[0005] WO 2009/1333466 provides methods and compositions for asymmetrically tagging a nucleic acid fragment using asymmetric adaptors.

[0006] Bennet et al. (2014) Biotechniques 56 (6) 289-300: "Library construction for ancient genomics; single strand or double- strand?" discloses a ligation-mediated PCR of restriction fragments from large DNA molecules.

SUMMARY

[0007] The present invention relates to use of an oligonucleotide vesicular adaptor for constructing a cyclic single-stranded library for nucleic acid sequencing with high efficiency.

[0008] The oligonucleotide vesicular adaptor comprises:

a 5' paired double-stranded region at a first terminal of the adaptor;
a 3' paired double-stranded region at a second terminal of the adaptor comprising a first strand and a second strand complementary with each other, wherein the first strand comprises an overhang at the 3' end thereof and the second strand comprises a phosphorylated base at the 5' end thereof so as to provide a sticky terminal and
a vesicular non-paired region between the 5' paired double-stranded region and the 3' paired double-stranded region, wherein the vesicular non-paired region comprises a first strand and a second strand non-complementary with each other and the first strand is of a length longer than that of the second strand.

[0009] The use of such an oligonucleotide vesicular adaptor according to the invention includes:

(a) ligating said oligonucleotide vesicular adaptor with a double-stranded DNA fragment to provide a structure K1-K2-K3 in which K1 and K3 each represent a vesicular adaptor ligated via the sticky terminal of said 3' paired double-stranded region;
(b) subjecting said double-stranded structure obtained in (a) to PCR amplification employing a first primer having the same sequence as at least a portion of the first strand of the vesicular non-paired region and a second primer specifically pairing with the second strand of the vesicular non-paired region to provide an amplified product for isolation of a single stranded DNA for cyclization.

[0010] In an embodiment of the present disclosure, the sticky terminal of the 3' paired double-stranded region has a single base tail.

[0011] In an embodiment of the present disclosure, the single base tail is thymine (T).

[0012] In an embodiment of the present disclosure, the vesicular adaptor is of a length of at least 20 nt, preferably 25 to 50 nt, and more preferably 30 to 45 nt.

[0013] In an embodiment of the present disclosure, the first strand of the vesicular non-paired region is longer than the second strand of the vesicular non-paired region by at least 5 to 30 nt.

[0014] In an embodiment of the present disclosure, the 5' paired double-stranded region also has a sticky terminal.

[0015] In an embodiment of the present disclosure, a sticky terminal of the 5' paired double-stranded region has 1 to 3 non-complementary bases.

[0016] In an embodiment of the present disclosure, the vesicular adaptor comprises a sense strand and an antisense strand, and is of a structure of formula I from the 5' terminal to the 3' terminal:

Y0-Y1-Y2 (I)

in which

Y0 represents the 5' paired double-stranded region, and is of a length of 10 to 15 nt, preferably 11 nt;
Y1 represents a non-paired double-stranded region, whose sense strand is of a length 5 to 30 nt longer than that of the antisense strand;
Y2 represents the 3' paired double-stranded region.

[0017] In an embodiment of the present disclosure, the vesicular adaptor has the following sequences:

5'-GTCCTAAGACCNGATCGGGCTTCGACTGGAGACTCCGACTT-3' (SEQ ID NO.:1)
5'-/phos/AGTCGGAGGCCAAGCGGTCTTAGGACAT-3' (SEQ ID NO.:2)

[0018] The present disclosure includes a kit for use of a vesicular adaptor in accordance with the invention. The kit includes:

a container;
an oligonucleotide vesicular adaptor for constructing a library in accordance with the invention, wherein the oligonucleotide vesicular adaptor is contained in the container;
a first primer having the same sequence as at least a portion of the first strand of the vesicular non-paired region;
a second primer, specifically pairing with the second strand of the vesicular non-paired region; and
an instruction.

[0019] In an embodiment of the present disclosure, the first primer is used as a sequencing primer.

[0020] In an embodiment of the present disclosure, the adaptor is contained in a container.

[0021] In an embodiment of the present invention
, a method for constructing a cyclic single-stranded library is provided. The method includes:

(a) end-repairing a double-stranded DNA fragment to obtain a double-stranded DNA fragment with blunt terminals;
(b) adding an adenine (A) base to each 3'-end of the double-stranded DNA fragment with the blunt terminals obtained in (a), to obtain a double-stranded DNA fragment with an A base at each 3'-end thereof;
(c) ligating an oligonucleotide vesicular adaptor as described above to each terminal of the double-stranded DNA fragment with the A base at each 3'-end thereof obtained in (b) to obtain a double-stranded DNA fragment ligated with the oligonucleotide vesicular adaptor at each terminal thereof;
(d) employing the double-stranded DNA fragment ligated with the oligonucleotide vesicular adaptor at each terminal thereof obtained in (c) as a template for PCR amplification with a pair of primers as described above so as to obtain a DNA amplified product, wherein one of the pair of primers is labelled with biotin;
(e) isolating the single-stranded DNA labelled with biotin from the amplified double-stranded DNA product obtained in (d) using beads coated with avidin through "avidin-biotin" combination thus obtaining the single-stranded DNA minus biotin for cyclization;
(f) subjecting the single-stranded DNA minus biotin obtained in (e) to cyclization in the presence of a cycling single-stranded molecule.

[0022] AS noted above, the double-stranded DNA fragment ligated with the oligonucleotide vesicular adaptor at each

terminal thereof obtained in (c) has a structure of formula III:

K1-K2-K3 (III)

in which,

K1 represents one vesicular adaptor as described above;
K2 represents an arbitrary DNA sequence (sequence of a fragment to be sequenced);
K3 represents another vesicular adaptor as described above,
in which K1 and K3 are connected to K2 respectively at two terminals of K2.

[0023] In an embodiment of the present disclosure, K2 is of a length of about 150 bp to about 250 bp.
[0024] In an embodiment of the present disclosure, the method further includes:

(g) digesting uncyclized DNAs contained in the mixture obtained in (f) with nucleases specifically digesting linear DNAs to obtain a pre-product; and
(h) purifying the pre-product obtained in (g) to obtain the cyclic single-stranded library.

[0025] In an embodiment of the present disclosure, the double-stranded DNA fragment in (a) is prepared by:

(a0) fragmenting a mRNA sample to obtain fragmented mRNAs; and
(a1) reverse transcribing the fragmented mRNAs to obtain cDNA amplified product as the double-stranded DNA fragments.

[0026] In an embodiment of the present disclosure, the double-stranded DNA fragment in (a) is obtained by fragmenting a DNA sample.
[0027] In an embodiment of the present disclosure, the avidin in (e) is streptavidin.
[0028] In an embodiment of the present disclosure, the pair of primers in (d) includes:

a forward primer:

5-/phos/AGACAAGCTCNNNNNNNNNNGATCGGGCTTCGACTGGAGAC (SEQ ID NO.:3); and
a reverse primer: 5-/bio/TCCTAAGACCGCTTGGCCTCCGACT (SEQ ID NO.:4),

in which,
5-/phos/ indicates that the 5' terminal nucleotide is modified by phosphorylation;
NNNNNNNNNN represents a tag sequence, where N represents adenine (A), thymine (T), cytosine (C) or guanine (G); and
5-/bio/ indicates that the 5' terminal nucleotide is marked with biotin.

[0029] In an embodiment of the present disclosure, the cycling single-stranded molecule in (f) is of a sequence: TCGAGCTTGTCTTCCTAAGACCGC (SEQ ID NO.: 5).
[0030] In an embodiment of the present disclosure, the nucleases used in (g) are exonucleases.
[0031] In an embodiment of the present disclosure, the nucleases used in (g) include a first exonuclease specifically digesting linear single-stranded DNAs and a second exonuclease specifically digesting linear double-stranded DNAs.
[0032] In an embodiment of the present disclosure, the nucleases include an enzyme mixture of Exo I and Exo III
The invention enables provision of a sequencing library for a high throughput sequencing platform that requires a cyclic single-stranded library, such as the Complete Genomics' sequencing platform.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033]

Fig. 1 is a flow chart showing the method for constructing a nucleic acid library according to an embodiment of the present disclosure;
Fig. 2 is a diagram showing the structure of a vesicular adaptor according to an embodiment of the present disclosure;
Fig. 3 is a diagram showing a result of a concentration of the purified PCR product detected by Agilent 2100 in nucleic acid library construction; and

Fig. 4 is an electrophoretogram of libraries detected by a 6% TBE denatured gel, where lanes 1 and 2 each represent a cyclic single-stranded library, while lane 3 represents low range ssRNA ladder.

DETAILED DESCRIPTION

[0034] The present inventors have for the first time developed a vesicular adaptor for efficiently constructing a nucleic acid sequencing library with high quality through extensive and in-depth studies and extensive screening. The experimental results show that, compared with sequencing data obtained from other nucleic acid sequencing library construction techniques, the nucleic acid sequencing library constructed with the vesicular adapter of the present disclosure has a higher quality and correlation, which can be used in the CG sequencing platform, thereby obtaining high authentic and reliable data without adverse influence on information analysis. Based on this, the present invention has been completed.

CG sequencing platform

[0035] For the CG sequencing platform, DNA nanoballs are embedded in a chip using high-density DNA nanochip technology, and bases in the sequence are read with combinatorial probe anchor ligation (cPAL) technology.

[0036] Cyclic single-stranded DNAs were obtained after library construction. A DNA nanoball (DNB), including more than 200 copies of cyclic single-stranded DNAs, was formed by rolling circle amplification, and then embedded into a hole in a chip using high-density DNAnanochip technology, with each hole only able to accommodate one DNAnanoball (as one DNAnanoball, once combined with the hole in the chip, will exclude the combination of other DNA nanoballs with the same hole). The occupancy rate of the DNA nanochip was over 90%, and each prepared DNA nanochip may accommodate 180 billion bases for imaging.

[0037] The cPAL technique uses probes marked with four different colors to read bases adjacent to the adaptor by at most 10 consecutive bases for each time. As each sequencing is independent from one another, i.e. the sequencing result is not affected by a previous sequencing result, error accumulation is thus avoided, which results in a high accuracy sequencing result with a base error rate as low as 1/100000. During sequencing, an anchor molecule is added to complementary pair with the adaptor, then the probes marked with four different colors are paired with corresponding bases of the template with the DNA ligases. The types of bases are determined by imaging fluorescent groups. Another advantage of cPAL technology is that, concentrations of probes and enzymes may be greatly reduced as the bases are read using a non-continuous and non-linkage combinatorial probe anchor ligation (cPAL) technology. Different from Sequencing by Synthesis, several bases may be read once in each cycle of cPAL, such that consumptions of sequencing reagents and imaging time may be both greatly reduced. Compared with the current popular next-generation sequencing technology, methods for constructing a library and sequencing the same according to embodiments of the present disclosure may obtain much more data while consuming fewer reagents.

Method for constructing a library

[0038] A RNA sample was digested with DNase I. The digested RNAs were purified with RNA clean magnetic beads. mRNAs from the total RNAs were isolated and purified with Oligo (dT) 25 magnetic beads, followed by fragmentation to obtain fragmented mRNAs. cDNAs were synthesized by reverse transcription of the fragmented mRNAs, and then end-repaired to form DNA fragments with blunt terminals to which were added A bases to obtain DNA fragments each with one A base at the 3 '-terminal thereof. The obtained DNA fragments each with one A base at the 3'-terminal thereof were ligated with vesicular adaptors to obtain DNA fragments each ligated with the vesicular adaptor at each terminal thereof, which were purified with magnetic beads and then amplified through polymerase chain reaction (PCR) where one primer used is marked with biotin. PCR product thus obtained was isolated by magnetic beads coated with streptavidin to obtain PCR single-stranded product, which was cyclized by bridge oligonucleotides and T4 ligases. Uncyclized PCR single-stranded product was enzymatically digested to obtain the cyclic single-stranded library.

Cyclic single-stranded library

[0039] The present disclosure also provides in embodiments a cyclic single-stranded library, which is suitable for sequencing and constructed by the above-described method for constructing a library.

[0040] In a preferred embodiment of the present disclosure, the present inventors have fully verified the stability, repeatability, and true reliability of the method of the present disclosure by exploring the optimum condition for constructing the library and comparing the results obtained under the optimum condition with that obtained by the other techniques. In addition, it is proved through several experiments with different samples that, the sequencing data obtained by the cyclic single-stranded library of the present disclosure is truly credible.

[0041] The advantages of the present disclosure lie in that:

(1) The vesicular adaptor for constructing the nucleic acid library is invented for the first time.
(2) With the use of a vesicular adaptor in embodiments of the present disclosure in the construction of the nucleic acid library, both the ligating efficiency and the efficiency of subsequent PCR are high and follow-up steps are few.
(3) The nucleic acid library in embodiments of the present disclosure may also be used in a sequencing platform which needs a cyclic single-stranded library.
(4) The method provided in embodiments the present disclosure is of high sequencing throughput, high accuracy and simple operation.
(5) The method provided in embodiments of the present disclosure is of high stability, repeatability and reliability.

[0042] The present disclosure will be further described in the following with reference to specific embodiments. It should be appreciated that these embodiments are merely used to illustrate the present disclosure and should not be construed to limit the scope of the present disclosure. Experimental methods in the following embodiments, not specifying the detailed conditions, will be carried out according to conventional conditions, such as described in Sambrook et al., Molecular Cloning: Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), or in accordance with conditions proposed by the manufacturer. Percentages and parts are by weight, unless otherwise stated.

Materials and methods

[0043] In the following embodiments, the reagent was prepared as follows: 5 × first strand buffer containing: 80-400 mM sodium chloride, 10-80 mM magnesium chloride, 200 mM to 300 mM Tris-HCl, phosphate, and water as solvent, with pH of 8.0-8.5. The standard substance, universal human reference RNA, was purchased from Agilent. Such RNA is a mixture from 10 kinds of human cell lines (breast cells, hepatoma carcinoma cells, cervical cells, embryonic cells, malignant glioma cells, melanoma cells, liposarcoma cells, lymphoma cells, leukemia T cells, and bone marrow B lymphocyte).

[0044] DNA fragments were purified by Ampure XP magnetic beads.

[0045] The materials used in embodiments of the present disclosure are all commercially available, unless specified otherwise.

Embodiment 1

Construction of RNA library with the use of the vesicular nucleotide adaptor

[0046] The specific procedures were carried out as follows (see the procedures shown in Fig. 1):

The specific procedures:

1. mRNA purification:

[0047]

1) Standard, universal human reference RNA (3 μg, Agilent), was added into an RNase-free tube and diluted into 50 μl with DEPC. The obtained mixture was denatured at 65 °C for 5 min subsequent to even mixing to degrade the secondary structure of RNA, then immediately placed on ice to obtain a RNA sample.
2) 15 μl Dynalbeads Oligo $(dT)_{25}$ magnetic beads were added into a non-stick-EP tube, washed twice with 100 μl binding buffer, then re-suspended in 50 μl binding buffer, followed by combining with the RNA sample obtained in 1), and finally stood still for 5 min at room temperature.
3) The non-stick-EP tube was placed on MPC (magnetic separator) for 2 min to remove the supernatant. The remaining magnetic beads were washed twice with 200 μl washing buffer. 50 μl binding buffer was added to a new non-stick-EP tube.
4) The EP tube (i.e. non-stick-EP tube in 3) containing magnetic beads was added with 50 μl 10mM Tris-HCl and heated at 80 °C for 2 min to elute the mRNAs from the magnetic beads. Then the non-stick-EP tube was quickly transferred onto the MPC. The mRNAs were transferred into the new non-stick-EP tube containing the binding buffer in 3), the obtained mixture was denatured at 65 °C for 5 min to degrade the secondary structure of mRNAs, then immediately placed on ice. In addition, 200 μl washing buffer was immediately added into the tube containing the remaining magnetic beads to wash the magnetic beads twice.
5) 100 μl mRNA sample was added with magnetic beads washed twice and then stood still for 5 min at room temperature. The EP tube was placed on MPC for 2min, the supernatant was carefully sucked out, and the remaining magnetic beads were washed twice with 200 μl washing buffer.

6) The EP tube containing magnetic beads was added with 17 μl 10mM Tris-HCl, then heated at 80 °C for 2 min to elute mRNAs from the magnetic beads. The EP tube was quickly placed on MPC. The eluent containing mRNAs was transferred into a new 200 μl PCR tube. About 16 μl mRNAs was recycled.

2. Fragmentation of mRNA and synthesis of a first strand

[0048] After combining with 3 μL 5× first strand buffer, the eluent obtained in the previous step was firstly incubated at 94 °C for 10 min followed by immediately placing on ice, then combined with 1 μl of random primers, and further incubated at 65 °C for 5min to degrade the second structure followed by placing on ice. A reaction mixture, formulated with 100 mM DTT (2 μl), 25 mM dNTP mixture (0.4 μl) and RNase inhibiter (0.5 μl), was added into the tube containing RNA, followed by mixed to be uniform and then stood still for 2 min at room temperature, then combined with 1 μl Superscript II (200U/μl) and water up to 25 μl. PCR reaction was performed in accordance with the following procedures:

Step 1	25°C	10min
Step 2	42°C	50min
Step 3	70°C	15min
Step 4	4 °C	hold

3. Synthesis of a second strand

[0049] After the above PCR reaction, the resulting reaction system was added to water up to 82.8 μl, then mixed with 10 μl 5 × second strand buffer and 1.2 μl 25 mM dNTP mixture in sequence to be uniform, followed by placing on ice for 5 min, and then mixed with 1 μl RNaseH and 5 μl DNA Pol I to be uniform. Such obtained reaction system for synthesizing a second strand was incubated at 16 °C for 2.5 h.

[0050] After the reaction was completed, the resulting double-stranded product was purified with Ampure XP magnetic beads, and the purified double-stranded product (DNAs) was dissolved in 50 μl EB buffer.

4. End-repairing

[0051] 50 μl solution containing double-stranded DNAs obtained in the previous step was successively added with 27.4 μl water, 10 μl 10X end repair buffer, 1.6 μl 25 mM dNTP mixture, 5 μl T4 DNA polymases, 1 μl Klenow DNA polymerase and 5 μl T4 PNK to form 100 μl reaction system which was incubated at 20°C for 30 min.

[0052] After the reaction was completed, the end-repaired product was purified with Ampure XP magnetic beads and then dissolved in 32 μl EB buffer.

5. Base A addition and adaptor ligation

[0053] 32 μl solution containing end-repaired DNAs obtained in the previous step was successively added with 5 μl A-tailing buffer, 10 μl 1 mM dATP and 3 μl Klenow exo (inhibiting activities of exonucleases for digesting from 3'-end to 5'-end) to form a reaction system of 50 μl, which was incubated at 37°C for 30 min.

[0054] After the reaction was completed, the base A-added product was purified with Ampure XP magnetic beads and then dissolved in 23 μl EB buffer.

[0055] 23 μl solution containing base A-added product obtained in the previous step was successively added with 25 μl 2X Rapid T4 DNA Ligase Buffer, 1 μl vesicular adaptor (with a structure as shown in Fig. 2) mixture (containing the vesicular adaptor in an amount of 50 μmol) and 1 μl T4 DNA Ligase to form 50 μl reaction system, which was incubated at room temperature for 15 min.

[0056] The adaptor sequence was as follows:

5'-GTCCTAAGACCNGATCGGGCTTCGACTGGAGACTCCGACTT-3' (SEQ ID NO.:1)
5'-/phos/AGTCGGAGGCCAAGCGGTCTTAGGACAT-3' (SEQ ID NO.:2).

[0057] After the reaction was completed, the ligation product was purified with Ampure XP magnetic beads, and then dissolved in 10 μl EB buffer.

6. PCR amplification and purification

[0058] 30 μl solution containing adaptor-ligated product obtained in previous step was successively added with 10 μl

5X Phusion buffer, 1 μl PCR Primer F (5-/phos/AGACAAGCTCNNNNNNNNNNGATCGGGCTTCGACTGGAGAC)(SEQ ID NO.:3), 1 μl PCR Primer R (5-/bio/TCCTAAGACCGCTTGGCCTCCGACT)(SEQ ID No.:4), 0.5 μl 25 mM dNTP mixture, 0.5 μl Phusion DNA polymerase and 7 μl water to form 50 μl reaction system, which was incubated in the PCR instrument according to the following procedures:.

a. 30 sec, 98°C
b. 15 cycles:

10 sec, 98°C
30 sec, 65°C
30 sec, 72°C

c. 5 min, 72°C
d. hold 4°C

[0059] After the reaction was completed, the amplified PCR product was purified with Ampure XP magnetic beads and then dissolved in 32 μl EB buffer. The concentration of the purified PCR product was detected with Agilent 2100, and the results are shown in Fig. 3.

7. Isolation of a single-stranded product

[0060]

7.1 Washing magnetic beads coated with streptavidin

30 μl magnetic beads coated with streptavidin (for each sample) was mixed with 90 μl to 150 μl 1X magnetic beads binding buffer to be uniform in a non-stick tube, which was then placed on a magnetic separator for still standing and adsorption. The non-stick tube was adjusted to be in such a direction that enables the magnetic beads to move forward and backward in the 1X magnetic beads binding buffer, followed by discarding the supernatant. After the direction adjustment step was repeated once, the non-stick tube was taken out from the magnetic separator, an 30 μl 1X magnetic beads binding buffer added, followed by standing still at room temperature.

7.2 After combining with water up to 60 μl, the purified PCR product obtained in the step 6 was firstly mixed with 20 μl 4X magnetic beads binding buffer to be uniform, and then transferred into the non-stick tube obtained in 7.1 which contained magnetic beads dissolved in 30 μl 1X magnetic beads binding buffer, followed by mixing to be uniform. Such a resulting 110 μl mixture was incubated at room temperature for 15 to 20 min, during which the mixture was flicked gently once to make it distribute evenly.

7.3 The non-stick tube after the step 7.2 was placed on the magnetic separator for 3 to 5 min, followed by discarding the supernatant. The remaining magnetic beads were washed twice with 1 ml 1X magnetic beads washing buffer as described in step 7.1.

7.4 The magnetic beads after the step 7.3 were evenly mixed with 78 μl 0.1M NaOH by blowing up and down to obtain a mixture, followed by standing still for 10 min and then placed on the magnetic separator for 3 to 5 min. 74.5 μl supernatant thus obtained was transferred into a new 1.5 ml EP tube.

7.5 37.5 μl 0.3M MOPS was added into the 1.5ml EP tube after the step 7.4, followed by mixed to be uniform, thereby obtaining 112 μl sample for use.

7.6 The 112 μl sample can be stored at -20 °C.

8. Cyclization of the single-stranded product

8.1 A primer reaction solution was formulated as follows about 5 min in advance:

ON1587 (TCGAGCTTGTCTTCCTAAGACCGC) (SEQ ID No.:5)

water	43 μl
20μM ON1587	20 μl
Total volume	63 μl

8.2 63 μl primer reaction solution obtained in the step 8.1 was mixed by shaking thoroughly, centrifuged, and then added into the sample of 112 μl obtained in step 7 (the starting amount n of the sample was critical and generally controlled within $100ng \leq n \leq 800ng$).

8.3 A ligase reaction solution was formulated as follows about 5 min in advance:

water	135.3 μl

(continued)

10x TA Buffer (LK1)	35 μl
100mM ATP	3.5 μl
600U/μl Ligase	1.2 μl
total	175 μl

8.4 175 μl ligase reaction solution obtained in step 8.3 was mixed by shaking thoroughly, centrifuged, and then added into the EP tube after step 8.2 which contained the primer reaction solution. A mixture thus obtained in this step was mixed by shaking for 10 s to be uniform, and then centrifuged.
8.5 The mixture obtained in step 8.4 was incubated in an incubator for 1.5 h at 37 °C.
8.6 After the reaction was completed, 10 μl resulting sample was detected by electrophoresis detection a 6% denatured gel, and the remaining sample in about 350 μl was allowed to the next enzymatic reaction.

9. Enzyme digestion

[0061]
9.1 An enzyme-digesting reaction solution was formulated as follows about 5 min in advance:

water	1.5 μl
10x TA Buffer (LK1)	3.7 μl
20U/μl Exo I	11.1 μl
200U/μl Exo III	3.7 μl
total	20 μl

9.2 20 μl enzyme-digesting reaction solution obtained in step 9.1 was mixed by shaking thoroughly, centrifuged, and then added into 350 μl sample obtained in the step 8.5 to obtain a mixture.
9.3 The mixture obtained in step 9.2 was mixed by shaking for 10 s to be uniform, and centrifuged, and then incubated in the incubator for 30 min at 37 °C.
9.4 After 30 min, the enzymatic reaction was stopped by adding 15.4 μl 500mM EDTA.
9.5 A sample obtained in step 9.4 was purified with 1.3X PEG32 magnetic beads/Tween 20 (or Ampure XP magnetic beads) as follows:
The sample obtained in step 9.4 was transferred into a 1.5 ml non-stick tube, and then combined with 500 μl PEG32 magnetic beads. The mixture thus obtained was left for binding at room temperature for 15 min, during which the mixture was mixed once by blowing up and down to be uniform.
9.6 The non-stick tube after step 9.5 was placed on the magnetic separator for 3 to 5 min, after which the supernatant was discarded, the remaining magnetic beads were washed twice with 700μl 75% ethanol, during each of which the non-stick tube was reversed forward and backward to enable the magnetic beads to move 2 to 3 times in the ethanol.
9.7 The magnetic beads after washing were air dried, and then re-dissolved in 40 μl 1X TE for 15 min, during which the mixture thus obtained was mixed once to be uniform.
9.8 Supernatant from the mixture obtained in the step 9.8 was transferred into a new 1.5 ml EP tube, the final product was quantified with Qubit™ ssDNA Assay Kit.
9.9 5 μl sample and 2μl low Range RNA ladder were respectively mixed with 5 μl 2x RNA loading buffer to be uniform in different PCR tubes, both of which were incubated at 95 °C for 2 min for denaturation in a PCR instrument, and quickly cooled on ice for 5 min. The resulting samples were detected with a 6% TBE denatured gel. The results are shown in Fig. 4.
9.10 Concentration standardization
[0062] The initial amount of the sample prepared with DNA nanoball (DNB) was uniformly adjusted to 7.5 fmol/μl in accordance with the concentration at which the single-stranded molecules were quantitatively detected.

Embodiment 2

Comparison of PCR efficiency of the vesicular adaptor in the library construction with that of other types of adaptors

[0063] The specific steps were as follows:
Steps same as those described in Embodiment 1 were carried out, where one adaptor was the vesicular adaptor, and the comparison adaptor was a matching adaptor. The PCR amplification and purification as described in step 6 were

completed, after which the amount of purified PCR product was detected.

[0064] Concentration of PCR template and recycling concentration were measured with Qubit dsDNA Assay Kit.

[0065] The experimental result is shown in Table

Adaptor	Matching adaptor	Vesicular adaptor
Amount of PCR template (ng)	10	10
Concentration of recycled product (ng/ul)	5.66	53
Total amount of recycled product (ng)	226.4	2120
PCR efficiency	1.366	1.709
Note : PCR efficiency=(total PCR yield/Initial amount of template) ×(1/cycles)		

[0066] It can be seen from above result that PCR efficiency of the vesicular adaptor is apparently higher than that of the matching adaptor.

SEQUENCE LISTING

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<110> MGI TECH CO., LTD

<120> VESICULAR ADAPTOR AND USES THEREOF IN NUCLEIC ACID LIBRARY CONSTRUCTION AND SEQUENCING

<130> P241685EPD1 /CI

<140> Divisional of EP14901593.5 based on PCT/CN2014/091852
<141> 2014-11-21

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Claims

1. Use of an oligonucleotide vesicular adaptor for constructing a cyclic single-stranded nucleic acid library wherein said adaptor comprises
a 5' paired double-stranded region at a first terminal of the adaptor;
a 3' paired double-stranded region at a second terminal of the adaptor, comprising a first strand and a second strand complementary with each other, wherein the first strand comprises an overhang at the 3' end thereof and the second strand comprises a phosphorylated base at the 5' end thereof so as to provide a sticky terminal and
a vesicular non-paired region between the 5' paired double-stranded region and the 3' paired double-stranded region, wherein the vesicular non-paired region comprises a first strand and a second strand non-complementary with each other and the first strand is of a length longer than that of the second strand,
and said use includes:

(a) ligating said oligonucleotide vesicular adaptor with a double-stranded DNA fragment to provide a structure K1-K2-K3 in which K1 and K3 each represent a vesicular adaptor ligated via the sticky terminal of said 3' paired double-stranded region;

(b) subjecting said double-stranded structure obtained in (a) to PCR amplification employing a first primer having the same sequence as at least a portion of the first strand of the vesicular non-paired region and a second primer specifically pairing with the second strand of the vesicular non- paired region to provide an amplified product for isolation of a single stranded DNA for cyclization.

2. The use according to claim 1, wherein the sticky terminal of the 3' paired double-stranded region of said oligonucleotide vesicular adaptor has a single base tail.

3. The use according to claim 2, wherein said single base tail is thymine (T).

4. The use according to any one of claims 1 to 3, wherein the first strand of the vesicular non-paired region is longer than the second strand of the vesicular non-paired region by at least 5 to 30 nt.

5. The use according to any one of claims 1 to 4, wherein the 5' paired double-stranded region also has a sticky terminal.

6. The use according to any one of claims 1 to 5, wherein the 5' paired double-stranded region has a sticky terminal of 1 to 3 non-complementary bases.

7. The use according to any one of claims 1 to 6, wherein said oligonucleotide vesicular adaptor comprises a sense strand and an antisense strand and is of a structure of formula I from the 5' terminal to the 3' terminal:

Y0-Y1-Y2 (I)

wherein

Y0 represents the 5' paired double-stranded region, and is of a length of 10-15nt, preferably 11nt;
Y1 represents a non-paired double-stranded region, whose sense strand is of a length 5-30nt longer than that of the antisense strand;
Y2 represents the 3' paired double-stranded region.

8. The use as claimed in any one of claims 1 to 7 wherein said first primer can also be used as a sequencing primer for sequencing of the cyclic single-stranded library following construction.

9. A use as claimed in any one of claims 3 to 8 wherein a method for constructing a cyclic single-stranded library is carried out, comprising:

(a) end-repairing a double-stranded DNA fragment to obtain a double-stranded DNA fragment with blunt terminals;
(b) adding an adenine (A) base to each 3'-end of the double-stranded DNA fragment with the blunt terminals obtained in (a) to obtain a double-stranded DNA fragment with an A base at each 3'-end thereof;
(c) ligating an oligonucleotide vesicular adaptor as defined in claim 3 to each terminal of the double-stranded DNA fragment with the A base at each 3'- end thereof obtained in (b) to obtain a double-stranded DNA fragment ligated with the oligonucleotide vesicular adaptor at each terminal thereof;
(d) employing the double-stranded DNA fragment ligated with the oligonucleotide vesicular adaptor at each terminal thereof obtained in (c), as a template for PCR amplification with said pair of primers so as to obtain a DNA amplified product, wherein one of the pair of primers is labelled with biotin;
(e) isolating the single-stranded DNA labelled with biotin from the amplified double-stranded DNA product obtained in (d) using beads coated with avidin through "avidin-biotin" combination, thus obtaining the single-stranded DNA minus biotin for cyclization;
(f) subjecting the single-stranded DNA minus biotin obtained in (e) to cyclization in the presence of a cycling single-stranded molecule.

10. The use according to claim 9, further comprising:

(g) digesting uncyclized DNAs obtained in (f) with nucleases specifically digesting linear DNAs to obtain a pre-product; and
(h) purifying the pre-product obtained in (g) to obtain the cyclic single-stranded library.

11. The use according to claim 10, wherein the nucleases used in (g) comprise a first exonuclease specifically digesting linear single-stranded DNAs and a second exonuclease specifically digesting linear double-stranded DNAs.

Patentansprüche

1. Verwendung eines vesikulären Oligonukleotid-Adapters für den Aufbau einer cyclischen einsträngigen Nukleinsäurebibliothek, wobei der Adapter folgendes umfasst:

eine 5' gepaarte doppelsträngige Region an einem ersten Ende des Adapters;
eine 3' gepaarte doppelsträngige Region an einem zweiten Ende des Adapters, welche einen ersten Strang und einen zweiten Strang umfasst, wobei die Stränge zueinander komplementär sind, wobei der erste Strang an dessen 3'-Ende einen Überstand umfasst, und wobei der zweite Strang an dessen 5'-Ende eine phosphorylierte Base umfasst, um ein klebriges Ende bereitzustellen; und
eine vesikuläre ungepaarte Region zwischen der 5' gepaarten doppelsträngigen Region und der 3' gepaarten doppelsträngigen Region,
wobei die vesikuläre ungepaarte Region einen ersten Strang und einen zweiten Strang umfasst, wobei die Stränge zueinander komplementär sind, und wobei der erste Strang eine Länge aufweist, die länger ist als die Länge des zweiten Strangs,
und wobei die Verwendung folgendes aufweist:

(a) Ligieren des vesikulären Oligonukleotid-Adapters mit einem doppelsträngigen DNA-Fragments, um eine Struktur K1-K2-K3 bereitzustellen, wobei K1 und K3 jeweils einen vesikulären Adapter darstellen, der über das klebrige Ende der 3' gepaarten doppelsträngigen Region ligiert ist;
(b) Unterziehen der in (a) erhaltenen doppelsträngigen Struktur einer PCR-Amplifikation, wobei ein erster Primer mit der gleichen Sequenz eingesetzt wird wie wenigstens ein Teil des ersten Strangs der vesikulären ungepaarten Region, und ein zweiter Primer, der spezifisch eine Paarung mit dem zweiten Strang der vesikulären ungepaarten Region bildet, um ein amplifiziertes Produkt zu erhalten zur Isolieren der einsträngigen DNA zur Cyclisierung.

2. Verwendung nach Anspruch 1, wobei das klebrige Ende der 3' gepaarten doppelsträngigen Region ein einzelnes Basenende aufweist.

3. Verwendung nach Anspruch 2, wobei das einzelne Basenende Thymin (T) ist.

4. Verwendung nach einem der Ansprüche 1 bis 3, wobei der erste Strang der vesikulären ungepaarten Region mindestens 5 bis 30 nt länger ist als der zweite Strang der vesikulären ungepaarten Region.

5. Verwendung nach einem der Ansprüche 1 bis 4, wobei die 5' gepaarte doppelsträngige Region ebenfalls ein klebriges Ende aufweist.

6. Verwendung nach einem der Ansprüche 1 bis 5, wobei die 5' gepaarte doppelsträngige Region ein klebriges Ende von 1 bis 3 nicht komplementären Basen aufweist.

7. Verwendung nach einem der Ansprüche 1 bis 6, wobei der vesikuläre Oligonukleotid-Adapter einen Sense-Strang und einen Antisense-Strang umfasst und eine Struktur der Formel I von dem 5'-Ende zu dem 3'-Ende aufweist:

Y0-Y1-Y2 (I),

wobei

Y0 die 5' gepaarte doppelsträngige Region darstellt und eine Länge von 10 bis 15 nt, vorzugsweise von 11 nt aufweist;
Y1 eine ungepaarte doppelsträngige Region darstellt, deren Sense-Strang 5 bis 30 nt länger ist als der Antisense-Strang;
Y2 die 3' gepaarte doppelsträngige Region darstellt.

8. Verwendung nach einem der Ansprüche 1 bis 7, wobei der erste Primer auch als ein Sequenzierungsprimer zur

Sequenzierung der cyclischen einsträngigen Bibliothek nach dem Aufbau verwendet werden kann.

9. Verwendung nach einem der Ansprüche 1 bis 8, wobei ein Verfahren für den Aufbau einer cyclischen einsträngigen Bibliothek ausgeführt wird, das folgendes umfasst:

(a) Reparatur der Enden eines doppelsträngigen DNA-Fragments, um ein doppelsträngiges DNA-Fragment mit stumpfen Enden zu erhalten;
(b) Hinzufügen einer Adenin (A)-Base zu jedem 3'-Ende des in (a) erhaltenen doppelsträngigen DNA-Fragments mit stumpfen Enden, um ein doppelsträngiges DNA-Fragment mit einer A-Base an jedem seiner 3'-Enden zu erhalten;
(c) Ligieren eines vesikulären Oligonukleotid-Adapters nach Anspruch 3 an jedem Ende des in (b) erhaltenen doppelsträngigen DNA-Fragments mit der A-Base an jedem seiner 3'-Enden, um ein doppelsträngiges DNA-Fragment zu erhalten, das an jedem seiner Enden mit dem vesikulären Oligonukleotid-Adapter ligiert ist;
(d) Einsetzen des in (c) erhaltenen doppelsträngigen DNA-Fragments, das an jedem seiner Enden mit dem vesikulären Oligonukleotid-Adapter ligiert ist als eine Vorlage für die PCR-Amplifikation mit einem Primerpaar nach Anspruch 8, um ein DNA amplifiziertes Produkt zu erhalten, wobei ein Primer des Primerpaares mit Biotin markiert ist;
(e) Isolieren der einsträngigen DNA, die mit Biotin markiert ist, aus dem in (d) erhaltenen amplifizierten doppelsträngigen DNA-Produkt unter Verwendung von durch "Avidin-Biotin"-Kombination mit Avidin überzogenen Beads, wodurch die einsträngige DNA minus Biotin zur Cyclisierung erhalten wird;
(f) Unterziehen der in (e) erhaltenen einsträngigen DNA minus Biotin einer Cyclisierung in Gegenwart eines cyclisierenden einsträngigen Moleküls.

10. Verfahren nach Anspruch 9, wobei dieses ferner folgendes umfasst:

(g) Verdauen in (f) erhaltener nicht cyclisierter DNA mit Nukleasen, die spezifisch lineare DNA verdauen, um ein Vorprodukt zu erhalten; und
(h) Aufreinigen des in (g) erhaltenen Vorprodukts, um die cyclische einsträngige Bibliothek zu erhalten.

11. Verfahren nach Anspruch 10, wobei die in (g) verwendeten Nukleasen eine erste Exonuklease umfassen, die spezifisch lineare einsträngige DNA verdaut, und eine zweite Exonuklease, die spezifisch lineare doppelsträngige DNA verdaut.

Revendications

1. Utilisation d'un adaptateur vésiculaire oligonucléotidique pour la construction d'une bibliothèque d'acides nucléiques simple brin cycliques, ledit adaptateur comprenant
une région à double brin jumelée 5' à une première terminaison de l'adaptateur ;
une région à double brin jumelée 3' à une seconde terminaison de l'adaptateur, comprenant un premier brin et un second brin complémentaires l'un de l'autre, le premier brin comprenant un surplomb à son extrémité 3' et le second brin comprenant une base phosphorylée à son extrémité 5' de sorte à fournir une terminaison collante ; et
une région vésiculaire non jumelée entre la région à double brin jumelée 5' et la région à double brin jumelée 3',
la région vésiculaire non jumelée comprenant un premier brin et un second brin non complémentaires l'un de l'autre et le premier brin ayant une longueur supérieure à celle du second brin,
et ladite utilisation comprenant les étapes consistant à :

(a) ligaturer ledit adaptateur vésiculaire oligonucléotidique avec un fragment d'ADN double brin pour obtenir une structure K1-K2-K3, K1 et K3 représentant chacun un adaptateur vésiculaire ligaturé par l'intermédiaire de l'extrémité collante de ladite région à double brin 3' jumelée ;
(b) soumettre ladite structure à double brin obtenue en (a) à une amplification par PCR en utilisant une première amorce ayant la même séquence qu'au moins une partie du premier brin de la région vésiculaire non jumelée et une seconde amorce se jumelant spécifiquement avec le second brin de la région vésiculaire non jumelée pour fournir un produit amplifié pour isoler un ADN simple brin à cycliser.

2. Utilisation selon la revendication 1, la terminaison collante de la région à double brin jumelée 3' dudit adaptateur vésiculaire oligonucléotidique ayant une queue de base unique.

3. Utilisation selon ladite revendication 2, ladite queue de base unique étant la thymine (T).

4. Utilisation selon l'une quelconque des revendications 1 à 3, le premier brin de la région vésiculaire non jumelée étant plus long que le second brin de la région vésiculaire non jumelée d'au moins 5 à 30 nt.

5. Utilisation selon l'une quelconque des revendications 1 à 4, la région à double brin jumelée 5' ayant également une terminaison collante.

6. Utilisation selon l'une quelconque des revendications 1 à 5, la région à double brin jumelée 5' ayant une terminaison collante de 1 à 3 bases non complémentaires.

7. Utilisation selon l'une quelconque des revendications 1 à 6, ledit adaptateur vésiculaire oligonucléotidique comprenant un brin sens et un brin antisens et ayant une structure de formule I de la terminaison 5' à la terminaison 3' :

Y0-Y1-Y2 (I)

Y0 représentant la région à double brin jumelée 5' et ayant une longueur de 10-15 nt, de préférence 11 nt ;
Y1 représentant une région à double brin non jumelée dont le brin sens est de 5 à 30 nt plus long que le brin antisens ;
Y2 représentant la région à double brin jumelée 3'.

8. Utilisation selon l'une quelconque des revendications 1 à 7, ladite première amorce pouvant également être utilisée comme amorce de séquençage pour le séquençage de la bibliothèque simple brin cyclique après la construction.

9. Utilisation selon l'une quelconque des revendications 3 à 8, un procédé de construction d'une bibliothèque simple brin cyclique étant réalisé, comprenant les étapes consistant à :

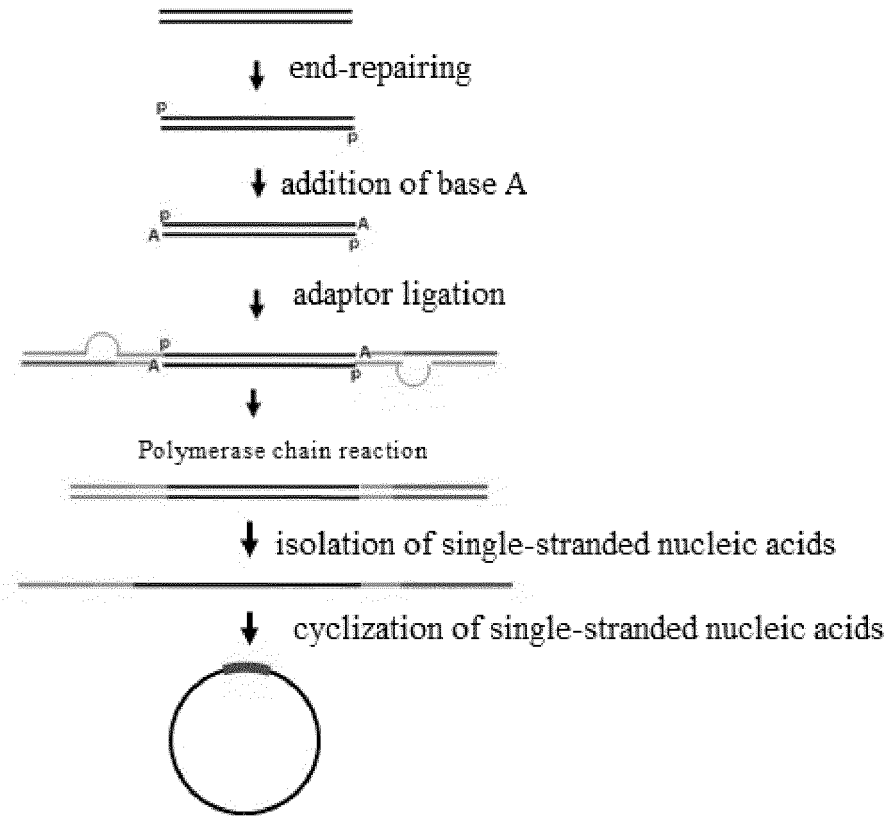
(a) réparer en bout un fragment d'ADN double brin pour obtenir un fragment d'ADN double brin avec des terminaisons émoussées ;
(b) ajouter une base d'adénine (A) à chaque extrémité 3' du fragment d'ADN double brin avec les terminaisons émoussées obtenues en (a) pour obtenir un fragment d'ADN double brin avec une base A à chaque extrémité 3' de celui-ci ;
(c) ligaturer un adaptateur vésiculaire oligonucléotidique selon la revendication 3 à chaque extrémité du fragment d'ADN double brin avec la base A à chaque extrémité 3' de celui-ci obtenue en (b) pour obtenir un fragment d'ADN double brin ligaturé avec l'adaptateur vésiculaire oligonucléotidique à chaque terminaison de celui-ci ;
(d) utiliser le fragment d'ADN double brin ligaturé avec l'adaptateur vésiculaire oligonucléotidique à chaque terminaison de celui-ci obtenu en (c), comme modèle pour l'amplification par PCR avec lesdites paires d'amorces de sorte à obtenir un produit amplifié par ADN, l'une de la paire d'amorces étant marquée avec de la biotine ;
(e) isoler l'ADN simple brin marqué à la biotine à partir du produit ADN double brin amplifié obtenu en (d) en utilisant des billes revêtues d'avidine par combinaison « avidine-biotine », obtenant ainsi l'ADN simple brin moins la biotine pour la cyclisation ;
(f) soumettre l'ADN simple brin moins la biotine obtenu en (e) à une cyclisation en présence d'une molécule simple brin cyclique.

10. Utilisation selon la revendication 9, comprenant en outre l'étape consistant à :

(g) digérer des ADN non cyclisés obtenus en (f) avec des nucléases digérant spécifiquement des ADN linéaires pour obtenir un pré-produit ; et
(h) purifier le pré-produit obtenu en (g) pour obtenir la bibliothèque simple brin cyclique.

11. Utilisation selon la revendication 10, les nucléases utilisées en (g) comprenant une première exonucléase digérant spécifiquement des ADN simple brin linéaires et une seconde exonucléase digérant spécifiquement des ADN double brin linéaires.

Figure 1



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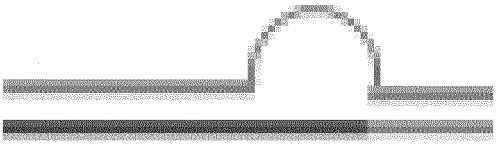

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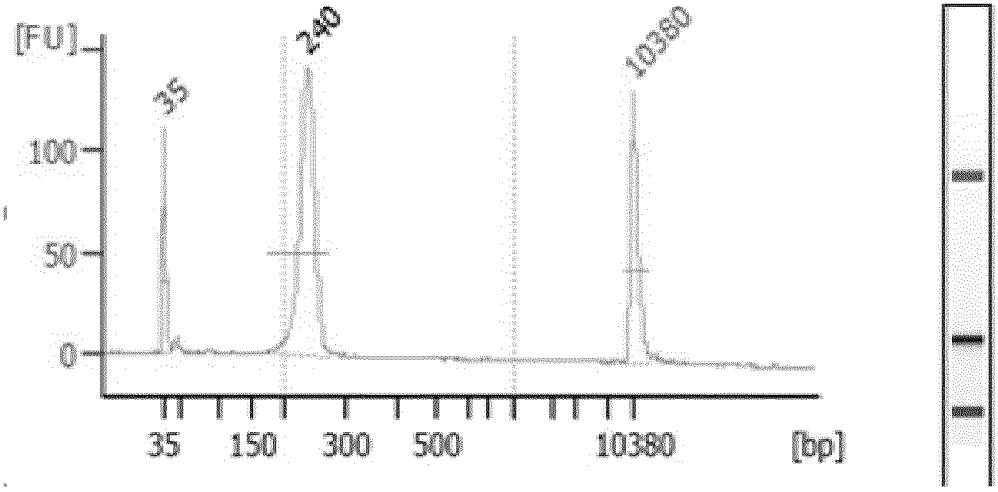
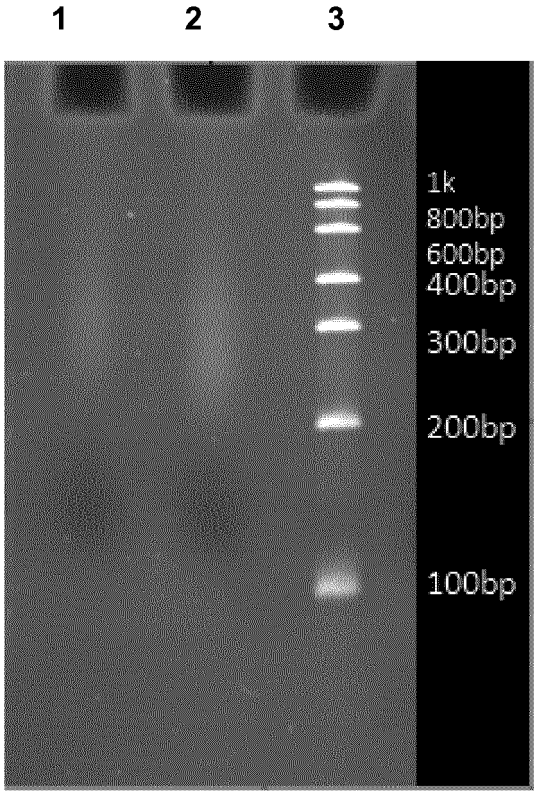


Figure 4



REFERENCES CITED IN THE DESCRIPTION

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